AFRL-ML-TY-TP-2006-4581

PREPRINT

BIOSENSOR FOR CONTINUOUS MONITORING OF ORGANOPHOSPHATE AEROSOLS

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December 2006

Submitted for publication in Biosensors and Bioelectronics.

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REPORT DOCUMENTATION PAGE

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1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-DEC-2006	Journal Article PREPRINT	01-DEC-2005 -- 01-NOV-2006

4. TITLE AND SUBTITLE

Biosensor for Continuous Monitoring of Organophosphate Aerosols

5a. CONTRACT NUMBER

F08637-03-C-6006

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

62102F

6. AUTHOR(S)

Luckarift, Heather R.; Greenwald, Roby; Bergin, Mike; Spain, Jim C., Johnson, Glenn R.

5d. PROJECT NUMBER

4915

5e. TASK NUMBER

L2

5f. WORK UNIT NUMBER

Q140LA62

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Applied Research Associates
P.O. Box 40128
Tyndall AFB, FL 32403

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

Air Force Research Laboratory
Materials and Manufacturing Directorate
139 Barnes Drive, Suite 2
Tyndall AFB, FL 32403-5323

10. SPONSOR/MONITOR'S ACRONYM(S)

AFRL/MLQL

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

AFRL-ML-TY-TP-2006-4581

12. DISTRIBUTION/AVAILABILITY STATEMENT

Distribution Statement A. Approved for public release; distribution unlimited.

13. SUPPLEMENTARY NOTES

Submitted for publication in Biosensors and Bioelectronics

14. ABSTRACT

An enzyme-based monitoring system provides the basis for continuous sampling of organophosphate contamination in air. The enzymes butyrylcholinesterase (BuChE) and organophosphate hydrolase (OPH) are stabilized by encapsulation in biomimetic silica nanoparticles, entrained within a packed bed column. The resulting immobilized enzyme reactors (IMERS) were integrated with an impinger-based aerosol sampling system for collection of chemical contaminants in air. The sampling system was operated continuously and organophosphate detection was performed in real-time by single wavelength analysis of enzyme hydrolysis products. The resulting sensor system detects organophosphates based on either enzyme inhibition (of BuChE) or substrate hydrolysis (by OPH). The system proved suitable for detection of a range or organophosphates including paraoxon, demeton-S and malathion. The detection limits of the IMERs for specific organophosphates are presented and discussed.

15. SUBJECT TERMS

enzyme immobilization, butyrylcholinesterase, organophosphate hydrolase, biosensor, impinger, aerosol sampling

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Glenn Johnson
U	U	U	SAR	22	19b. TELEPHONE NUMBER *(Include area code)*

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Standard Form 298 (Rev. 8/98)
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BIOSENSOR FOR CONTINUOUS MONITORING OF ORGANOPHOSPHATE AEROSOLS

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Abstract

An enzyme-based monitoring system provides the basis for continuous sampling of organophosphate contamination in air. The enzymes butyrylcholinesterase (BuChE) and organophosphate hydrolase (OPH) are stabilized by encapsulation in biomimetic silica nanoparticles, entrained within a packed bed column. The resulting immobilized enzyme reactors (IMERs) were integrated with an impinger-based aerosol sampling system for collection of chemical contaminants in air. The sampliug system was operated continuously aud organophosphate detection was performed in real-time by single wavelength analysis of enzyme hydrolysis produets. The resnlting sensor system detects organophosphates based on either enzyme inhibition (of BuChE) or substrate hydrolysis (by OPH). The systeun proved suitable for detection of a range of organophosphates inclnding paraoxon, demeton-S and malathion. The detection limits of the IMERs for specific organophosphates are presented and discussed.

Keywords: Enzyme Immobilization: Butyrylcholinesterase; Organophosphate Hydrolase; Biosensor: Impinger; Aerosol sampling

1. Introduction

Organophosphates (OPs) are used throughout the world as pesticides and insecticides. OPs are also toxic to many other organisms, including humans, due to their often irreversible inhibition of essential enzymes of the central nervous system. The potency of OPs has also led to their use as chemical warfare agents (Bajgar, 2004; Wiener and Hoffman, 2004). Currently, the detection of OP exposure is mostly retrospective, because many OPs exist as atmospheric particulate matter not easily detectable by human senses. The threat of terrorist activity is often used to emphasize the importance of sampling and analysis of airborne contaminants, yet less sinister circumstances can cause public health issues as well. For example, the ventilation of buildings using outdoor air creates a situation whereby building occupants can be exposed to OPs both by incidental release (e.g. pesticide spraying) as well as a direct targeted attack. Therefore, there is an urgent need for aerosol sampling devices that provide an early warning of OP chemical agent release before the resultant contamination of a buildings air supply.

Detection of OPs in air is currently performed by chromatography coupled with mass selective detectors or various types of spectroscopy (Staaf and Ostman, 2005; Bjorklund et al., 2005; Sanchez et al., 2003). Such techniques are time consuming, expensive and often require highly trained personnel, making them impractical for continuous monitoring. The enzymes acetylcholinesterase (AChE), butyrylcholinesterase (BuChE) and organophosphate hydrolase (OPH) have received much attention as alternatives for the detection of OPs and have been demonstrated in a range of

amperometric, potentiometric, conductometric and optical formats (Andreescu and Marty, 2006; Walker and Asher, 2005; Mulchandani et al., 2001; Simonian et al., 2001; Mulchandani et al., 1998; Wang et al., 2003; Singh et al., 1999). Detection using BuChE is based upon enzyme inhibition: OPs block the enzyme activity of BuChE leading to a decrease in response proportional to OP concentration. In contrast, detection using OPH is based upon hydrolysis of OP substrates. OPH hydrolyzes a range of OPs including; pesticides such as parathion and malathion and chemical warfare agents such as soman, sarin and VX (Dumas et al., 1989; Di Sioudi et al., 1999; Lai et al., 1995; Rastogi et al., 1997). The products of hydrolysis can be monitored spectrophotometrically or electrochemically. Because OPs are substrates for OPH, catalysis leads to a direct determination of analyte and the signal generated is directly proportional to the concentration of OP.

One of the primary limitations of many biologically-based detection systems is the instability of the biological component. We have recently demonstrated that silicification provides a biocompatible and simple method for enzyme immobilization (Luckarift et al., 2004). Silicification refers to the process that certain organisms use to form hard silica skeletons by deposition of inorganic minerals. The silicification process can be adapted in *vitro* to entrap enzymes of interest within the resulting silica matrix. The silica immobilization method proved suitable for the encapsulation of butyrylcholinesterase (BuChE) with high retention of enzyme activity (>90%). Further modification of the procedure allowed the entrainment of the silica-immobilized enzymes within a fixed bed column; making them amenable to continuous flow systems (Luckarift et al., 2006). In this study, we compare the applicability of silica-immobilized BuChE

and OPH for enzyme-based detection of OP contaminated air. The system provides the potential for development of continuous monitoring of air for OP contamination and integration of the detection with an alarm system. A system is envisaged for example whereby an air conditioning supply could be controlled in response to a positive signal.

2. Materials and methods

2.1 Materials

Butyrylcholinesterase (E.C.3.1.1.8; Equine serum, ≈ 50% protein and activity of 1,200 Units per mg protein) was purchased from Sigma-Aldrich and dissolved in cholinesterase specific buffer (Luckarift et al., 2004). The synthetic peptides R5 (SSKKSGSYSGSKGSKRRIL), and the R5-His-tagged variants were purchased from New England peptide (Gardner, MA). Demeton-S was obtained from ChemService (West Chester, PA). All other chemicals were of analytical grade and obtained from Sigma-Aldrich (St. Louis, MO). OPH was kindly provided by Dr. James Wild of Texas A and M University.

2.2 Air Sampler Design: A schematic diagram of the sampling system is illustrated in Figure 1. Liquid samples were aerosolized using a Micro-Mist nebulizer manufactured by Hudson RCI. These nebulizers were loaded with 1 ml of solution and operated at an air flow rate of approximately 4 L/min for 4 minutes. At the end of 4 minutes, the nebulizer was switched off and the residual volume of sample remaining in the nebulizer was measured to determine the volume of aerosolized sample. Downstream of the nebulizer, aerosolized samples were diluted with approximately 8 L/min of filtered room air. The

combined flow of sample and diluent air was collected in a SKC™ Biosampler impinger. This device employs three glass jets to impact a gas or aerosol sample into a liquid reservoir. The airflow through the impinger jets was maintained at sonic speed by providing a stable flow rate of 12 L/min. At this gas flow rate, the impinger has been documented by its manufacturer to have an aerosol collection efficiency of 80% for 0.3 μm particles to ~100% for particles greater than 1.0 μm. For these experiments, the liquid reservoir in the impinger was composed of phosphate buffer (25mM, pH 7.0, 10mM $MgSO_4$, 126 μM 5,5'-dithiobis (2-nitro-benzoic acid [Ellman's reagent]) and was continuously circulated at a flow rate of ~ 20 ml/min using a Fluid Metering, Inc. Model QV piston pump. After the aerosolized sample has been entrained into the circulating liquid stream, the mobile phase passes through a passive debubbling device to remove air bubbles. The bubble-laden flow is diverted to waste while the bubble-free flow is directed through the IMER columns and into a single wavelength absorbance detector. This flow rate is maintained at 2 ml/min by a Fluid Metering, Inc. Model QV piston pump positioned between the debubbler and the columns so that the sample is under positive pressure as it is pushed through the column and negative pressure as it is pulled through the detector. Downstream of the detector, the sample is diverted to waste.

The relationship between the concentration of a compound in the air and that in the impinger liquid can be expressed as $C_A = C_l \times (Q_l / Q_A)$. Where C_A is the atmospheric concentration of the organophosphate of interest, C_l is the concentration in the impinger liquid, Q_l is the liquid flow rate through the impinger and Q_A is the air flow rate within the impinger. Hence for a measured liquid concentration the atmospheric concentration can be determined.

A sample calculation for determining the atmospheric concentration of paraoxon (1ml of 1 mM atomized over 4 min., MW = 275) is demonstrated below;

Equation 1: Atomizer generation rate = (0.1 x 10^{-3} moles/L) (1ml/4min) (L/1000ml) = 2.5 x 10^{-7} moles/min

Eqnation 2: C_{AIR} = (2.5 x 10^{-7} moles/min) (min/12 L_{AIR}) = 2.1 x 10^{-8} moles/L_{AIR} = 2.1 x 10^{-5} moles/m^3_{AIR}

Equation 3: Mass concentration; C_{AIR} = (2.1 x 10^{-5} moles/m^3_{AIR}) (275 g/mole) (10^6 µg/g) = 5729 µg/m^3

Equation 4: Estimation of the volume concentration using ideal gas law:

ppm = {C (µg/m^3) RT/PM} x 10^{-3} = {(5729 µg/m^3) (0.08208 ATM · m^3/ Kg · mol K) (300K)} / {(1 ATM) (275 kg/kg · mole) = 0.513 ppm = 513 ppb

2.3 IMER preparation and chromatography conditions

Immobilized enzyme reactor columns (IMERs) were prepared as described previously (Luckarift et al., 2006), with either BuChE (160 Units) or OPH (0.66 mg). In preliminary experiments, IMERs were connected to an Agilent 1100 series liquid chromatography system and liquid samples (20 µl) were injected directly via an auto injection system. For aerosol sampling, the IMER columns were integrated with a Biosampler Impinger (SKC, Eighty Four, PA) and attached to a Hewlett Packard 1050 series single wavelength detector (Figure 1). Phosphate buffer (25 mM, pH 7.0, 10 mM $MgSO_4$, 126 µM 5,5'-dithiobis (2-nitro-benzoic acid [Ellman's reagent) was used as the mobile phase throughout unless otherwise stated.

OPH-IMERs: OPH-catalyzed hydrolysis of OPs was monitored continuously at 412 nm for malathion and demeton-S and 400 nm for paraoxon. Ellman's reagent within the mobile phase reacts with free thiols generated by hydrolysis of malathion or demeton-S to produce a detectable chromophore at 412 nm. Detection of paraoxon is by direct determination of the hydrolysis product, p-nitrophenol at 400 nm. Signal intensity for all

substrates was recorded as the product peak area (Figure 2a). Goodness of fit (r^2) for linear regression lines was determined using GraphPad Prism software (v3.02).

BuChE-IMERs: Butyrylthiocholine iodide (BuCh-I) (20 μM in phosphate buffer) was pumped continuously through the column. BuChE-catalyzed hydrolysis of BuCh-I was monitored continuously at 412 nm. For inhibition experiments, a stock solution of inhibitor was prepared in ethanol and diluted into phosphate buffer to give a range of inhibitor concentrations (0.1 – 5 mM). The degree of inhibition was determined by calculating the change in peak height compared to initial steady state absorbance (Figure 2b).

BuChE Reactivation: After incubation with inhibitors. BuChE-IMERs were reactivated by flowing 4 column volumes (20 ml) of pyridine-2-aldoximine (PAM) (5 mM) through the column at 4 ml/min. followed by 4 column volumes of buffer (containing 126 μM Ellman's reagent and 20 μM BuCh-I) at 4 ml/min. Long-term reactivation studies were performed on an Agilent 1100 series liquid chromatography system with buffer as one mobile phase and the PAM reactivator (5mM) as a second mobile phase.

3. Results and discussion

3.1 Enzyme based IMER biosensors

Immobilized enzyme reactor (IMER) biosensors were constructed with either OPH or BuChE as the immobilized enzyme component as described previously for BuChE (Luckarift et al., 2006). Briefly, IMERs are prepared using silica-encapsulation *in situ* via histidine-tag attachment of the silica/enzyme nanocomposites to a packed column

by metal affinity binding. Effective loading and retention of the enzyme activity was achieved. The protein concentration of the eluate indicated greater than 90% loading efficiency; in agreement with previous studies for BuChE (Luckarift et al., 2006). The IMER columns gave stable and reproducible conversion of substrate over a 24 hour period. In addition, the IMER columns could be stored at 4°C and reused over a period of 5 days with no loss in activity (data not shown).

3.2 Detection of OPs by OPH-catalyzed hydrolysis in liquid

Detection using the biosensor containing OPH is based on the hydrolysis of OP substrates and the concentration of product formed. Three OP pesticides were selected as representative substrates; paraoxon is representative of OPs with phosphotriester bonds (P-O bond), demeton-S and malathion are representative of phosphonothioate pesticides (P-S bond). Initially the IMERs containing silica-immobilized OPH were tested by direct injection of paraoxon in the liquid phase. OPH catalyzed the hydrolysis of paraoxon to yield PNP and produced a concentration-dependent response to paraoxon at a range of flow rates (Figure 3). A more rapid signal response was observed by increasing the liquid flow rate through the impinger, but there was a concurrent decrease in signal intensity and hence the sensitivity of the system resulted from the reduced residence time. Detection of paraoxon in the absence of the OPH-IMER was minimal (less than 10 % relative signal intensity).

Detection of hydrolysis products by spectrophotometric analysis is limited to substrates that produce a strong chromophore. Inclusion of Ellman's reagent within the reaction, however, enhances the detection of free thiol groups generated by the hydrolysis

of P-S bonds (as in demeton-S and malathion) (Lai et al., 1995; Rastogi et al., 1997). Detection of demeton-S produced a linear response in the range 0.01 – 0.2 μmol (r^2 = 0.9816) and the signal intensity was significantly enhanced compared to that in an enzyme-free column (Figure 4). Malathion was also detectable following OPH catalysis and produced a linear response in the same substrate concentration range (0.01 – 0.2 μmol; r^2 = 0.9998), but the signal was not significantly different from an enzyme-free control at low concentrations (Figure 4).

3.3 Aerosol Sampling System configuration and characterization

The applicability of the IMERs connected to an aerosol sampling system was investigated. Initially, a control column containing no enzyme was connected to the system shown in the schematic (Figure 1) to determine the efficiency and reproducibility of aerosol collection. The impinger device entrains contaminant particles into a fixed volume of buffer. The liquid flow rate into the impinger is controlled at a rate that can be adjusted to vary the collection efficiency and dilution factor. Aerosol samples collected within the impinger are pumped through the IMER columns and into a single-wavelength detector. p-Nitrophenol (PNP) was selected as a model compound for calibration because it contains a strong chromophore that can be detected at 400nm. A linear response for PNP was observed in the range 0.1 – 5 μmol (r^2 =0.994). The response time was approximately 6 minutes and was a function of the combined residence time of the impinger, debubbler and IMER column. System parameters such as the impinger liquid flow rate can be modified to change the response time of the instrument.

3.4 Detection of OPs in air by OPH-catalyzed hydrolysis

The OPH-IMER was connected to the impinger aerosol sampling system to determine the efficiency and reproducibility of the system during continuous operation. Paraoxon was aerosolized via an atomizer at a range of concentrations and the corresponding signals demonstrated first order reaction kinetics in the range 0 to 2.5 ppm (r^2 =0.994). The signal intensity for detection of PNP as a product of OPH hydrolysis was in agreement with the signal observed for PNP provided directly to the system, confirming complete hydrolysis of paraoxon at the concentrations tested (Figure 5). Malathion and demeton-S produced lower responses than paraoxon (Table 1a). The difference between sensor responses of various OP substrates varied considerably and is attributed to variations in the substrate specificity of OPH as noted previously (Sioudi et al., 1999; Dumas et al., 1989). The K_m value for OPH catalyzed hydrolysis of demeton-S, for example, is approximately 10-fold higher than for paraoxon (Lai et al., 1995).

3.5 Detection of OPs by BuChE inhibition in air

The activity of BuChE can be determined by monitoring the hydrolysis of butyrylthiocholine iodide (BuCh-I) and measuring the concurrent formation of product upon reaction with Ellman's reagent to produce a detectable yellow anion (Lockridge et al., 2006 and references therein). OPs inhibit the hydrolysis of BuCh-I producing a decrease in signal intensity that is directly proportional to inhibitor concentration. Buffer was pumped continuously through the impinger at a fixed flow rate (20 ml/min) to give a constant substrate concentration of BuCh-I (20 μM). This concentration yielded steady state continuous hydrolysis of BuCh-I to thiocholine. Paraoxon, demeton-S and

malathion were investigated as representative cholinesterase inhibitors. All substrates were delivered to the aerosol sampling system via an atomizer and the inhibition of the steady state signal was measured. The inhibition of BuChE by paraoxon produced a second order kinetic response within the range 5-50 ppb (Figure 5b). The signal response was reproducible with standard deviations of <10% and was reproducible among three independent OPH-IMERs (data not shown). Inhibition of BuChE by paraoxon was linear at low concentrations but became saturated and lost linearity at concentrations above 25 ppb paraoxon. The sensitivity of the BuChE-IMER was in the order paraoxon>malathion>demeton-S (Table 1b). The sensitivity for the BuChE-IMER is in agreement with known inhibition of acetylcholinesterase enzymes by OPs (Simonian et al., 2001).

3.6 Biosensor reactivation and stability

BuChE becomes inactivated when exposed to OPs (Gulla et al., 2002; Mulchandani et al, 2001). In order to evaluate the BuChE-IMER it was necessary to reactivate the column and determine the recovered activity. Following inhibition of the BuChE-IMER, the column was treated with pyridine-2-aldoxime (PAM); a known reactivator of OP-inhibited cholinesterase enzymes (Kovarik et al., 2004; Gulla et al., 2002). BuChE was to some extent irreversibly inhibited by paraoxon and demeton-S and there was a continuous decrease in enzymatic activity during repeated use of the IMER columns for inhibition experiments, even with use of the reactivator (Figure 6). Loss of activity, however, was only detected when a saturating substrate concentration is used for activity measurements. At lower substrate concentrations, enzyme activity (> 70%) is

retained providing reproducible and stable activity measurements following reactivation and reuse of the IMER columns for the entirety of the experiment (Figure 6).

Despite the need to reactivate the BuChE biosensor, both OPH and BuChE biosensors were reusable over a number of samples with no loss in activity. An important characteristic of both the OPH and BuChE biosensors was the stability and reproducibility of the signal response over successive samples (Figure 7). The stability of the signal also indicates no significant carryover of sample between subsequent analyses, which has been observed as a problem of inhibition-based biosensors.

4. Conclusion

The integration of IMER columns with an aerosol sampling system proved suitable for the detection of OPs and provided reliable and reproducible data for OPs including paraoxon, demeton-S and malathion. In the case of the OPH-IMERs, a difference between sensor responses with various OP substrates is directly attributed to variations in the substrate specificity of OPH (Di Sioudi et al., 1999; Dumas et al., 1989). The sensitivity of the systems for demeton-S and malathion differed by an order of magnitude relative to detection of paraoxon for both OPH and BuChE detection systems. Increased sensitivity and a correspondingly lower detection limit for the OPH-IMER can potentially be attained by utilizing site-directed mutants of OPH with variations in substrate specificity. For exaunple, OPH has low catalytic activity towards demeton-S. Genetically modified OPH, however, shows increased catalytic activity towards P-S bonds which could be utilized to enhance the biosensor response (Lai et al., 1996; Simonian et al., 2004)

For a continuous air-sampling detector it is not necessary to immediately identify the agent, but simply to provide an early warning. The addition of a high-pressure liquid chromatography column in-line with the current detection system would allow the discrimination and quantification of specific OPs detected by the system. This integration would provide an initial detection response that may be followed by a more detailed examination of the nature of the contaminant. The system may therefore warn against any compound that can act as a cholinergic neurotoxin, irrespective of its origin. This system is adaptable for example, to monitoring pesticide concentrations in public water supplies or integration with the HVAC intake to a building.

The sensitivity aud detection limits demonstrate the applicability of the system. but significant optimization of the system will be required to enhance applicability. The system still has inherent limitations, for example the OPH-IMER does not require a continuous supply of enzyme substrate, but the BuChE-IMER does. With additional optiuization, the biosensor system described may find application for real-time detection of chemical agents under environmental conditions. The detection of OPs by the aerosol sampling system is however, simple, rapid and provides a direct measurement applicable to kinetic response measurements.

Acknowledgement

This work was supported by funding from the U.S Air Force Office of Scientific Research. HRL was supported by Oak Ridge Institute for Science and Education (U.S. Department of Energy).

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Fig. 1. Schematic of an organophosphate measurement system

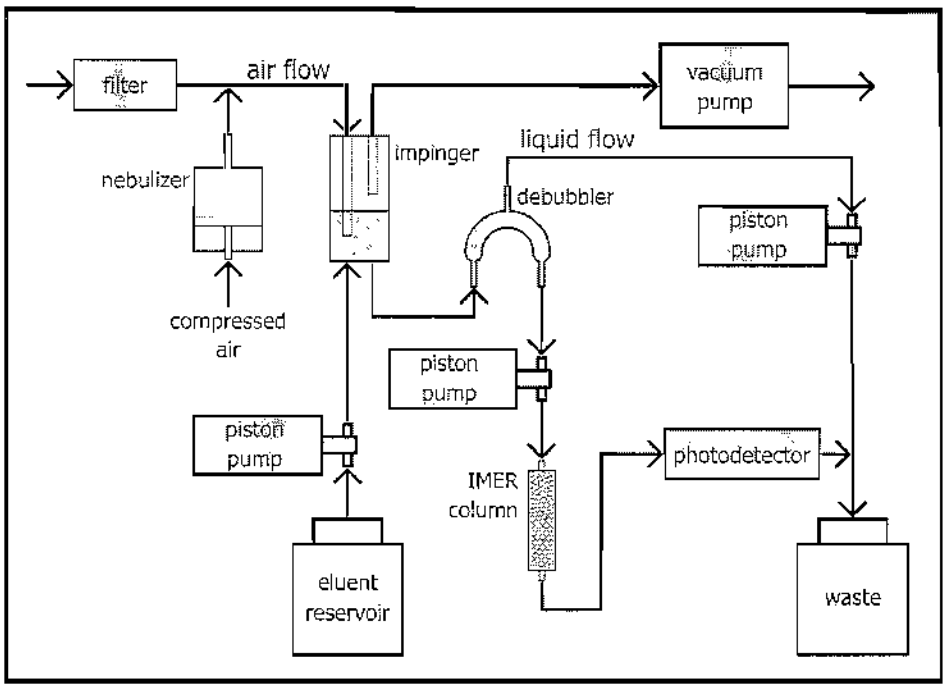

Figure 2. Typical Signal responses for paraoxon from the aerosol sampling system

A) Hydrolysis of paraoxon by OPH generates a signal measured by peak area (hatched area under peak); B) Inhibition of BuChE by paraoxon generates a signal measured by decrease in peak height from steady state.

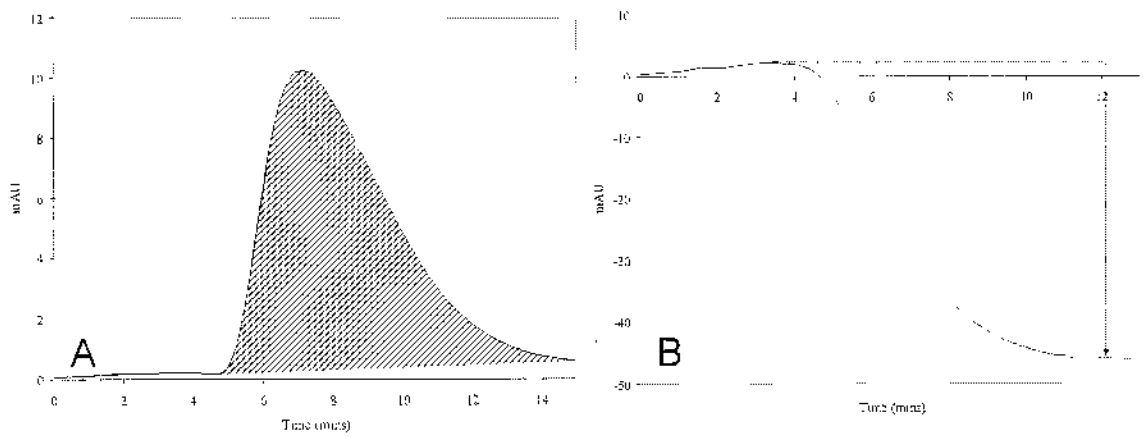

Figure 3. OPH catalyzed hydrolysis of Paraoxon as a function of flow rate

Paraoxon was injected to OPH columns at a range of concentrations and signal intensity (peak area at 400 nm) of the hydrolysis product was measured at a range of flow rates; (■) 1 ml/min, (▲) 1.5 ml/min, (●) 2 ml/min

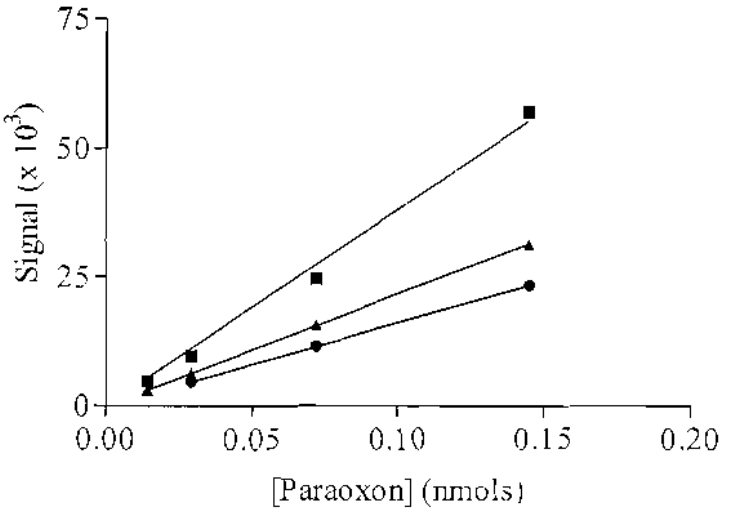

Figure 4. Detection of demeton-S and malathion by OPH-catalyzed hydrolysis

Substrate (Demeton-S and Malathion) was injected into OPH columns at a range of concentrations and signal intensity of hydrolysis products were measured compared to an (enzyme-free) control column. Ellman's reagent was present in the mobile phase (126 μM). OPH-IMER column (■), Enzyme-free control column (○).

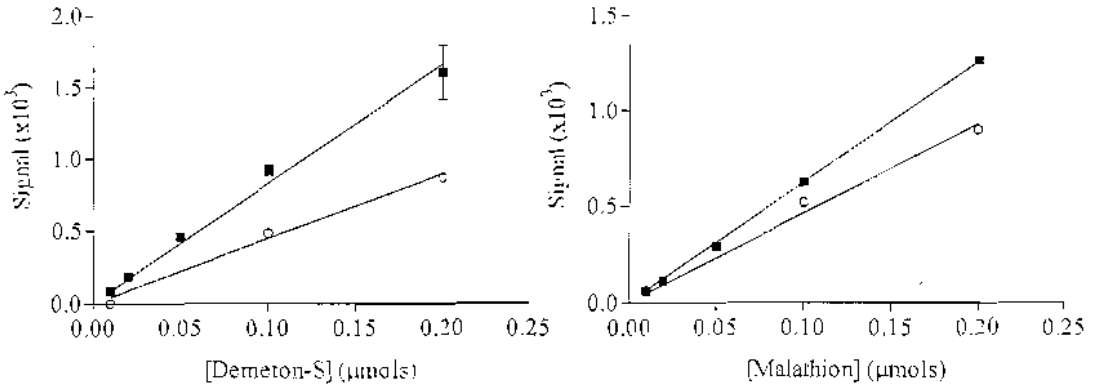

Figure 5a. Detection of paraoxon by hydrolysis using the aerosol sampling system

A) OPH-catalyzed hydrolysis of paraoxon produces a signal response as a function of paraoxon concentration, B) Linear correlation of substrate concentration to signal (peak area): p-nitrophenol (PNP) was detected directly through a blank column (○) or as a product of paraoxon hydrolysis by OPH-IMER (■)

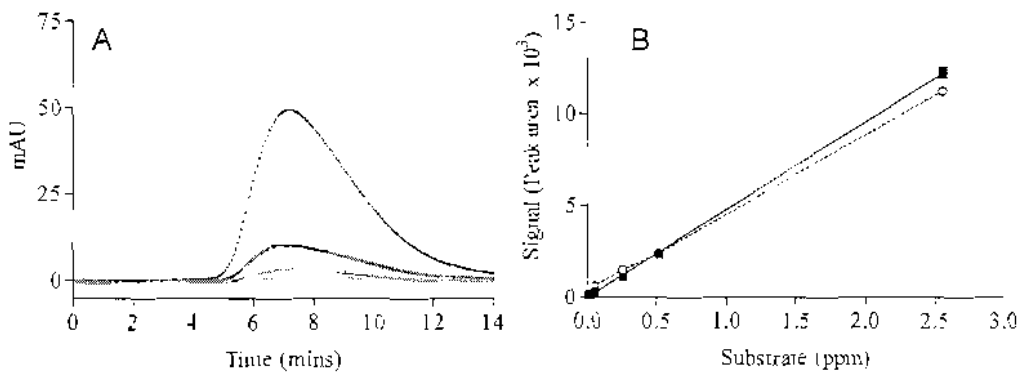

Figure 5b. Detection of paraoxon by inhibition using the aerosol sampling system

A) BuChE inhibition by paraoxon produces a signal response as a function of paraoxon concentration, B) Correlation of substrate concentration to sigual (decrease in peak height)

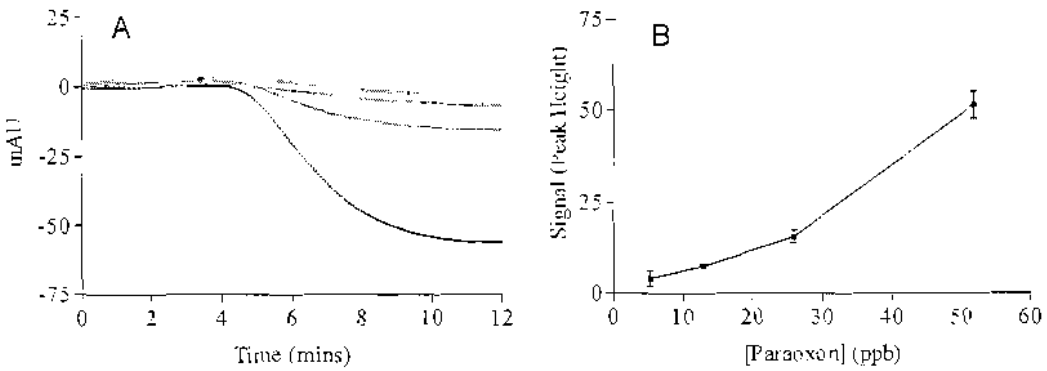

Figure 6. Reactivation of BuChE following inhibition by demetou-S and paraoxon

Paraoxon and Demeton-S were injected to BuChE columns (20 µl of 5mM). Following reactivation with pyridine-2-aldoxime (4 column volumes of 5mM at 4ml/min). BuCh-I was supplied at high/saturated (20 µl of 20 mM) and low (2 µl of 20 mM) substrate concentrations to measure retention of enzyme activity. Paraoxon; [high BuCh-I] (▲), [low BuCh-I] (△). Demeton-S; [high BuCh-I] (■), [low BuCH-I] (□)

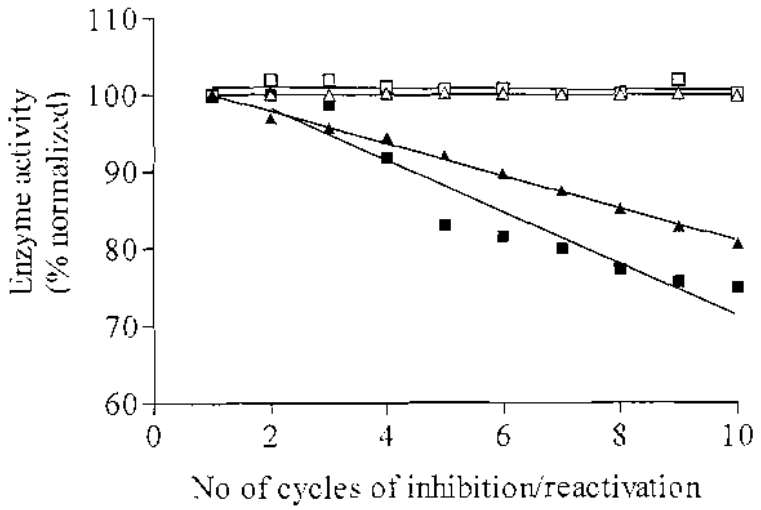

Figure 7. Stability of the signal response to paraoxon over successive measurements

A) Response of the OPH-IMER biosensor; and B) Response of the BuChE-IMER biosensor to suecessive changes in paraoxon concentration.

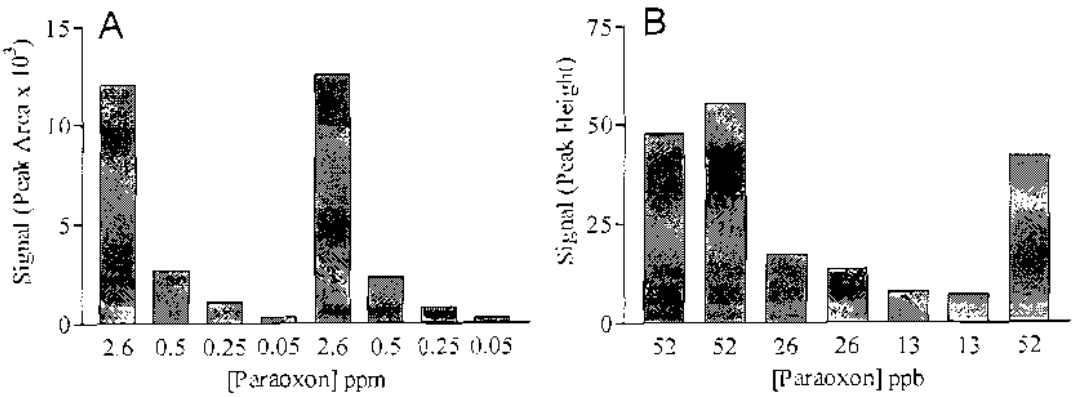

Table 1. Signal intensity for a range of OPs in the aerosol sampling system

A) OPH-IMER column

	Paraoxon		Demeton-S		Malathion	
	513 ppb	*2.6 ppm*	*513 ppb*	*2.6 ppm*	*513 ppb*	*2.6 ppm*
Signal	2329 ± 346	12254 ± 402	40.5 ± 23	187.2 ± 44	30.1 ± 1.8	ND
*Intensity	100%	100%	1.7%	1.5%	1.3%	-

B) BuChE-IMER column

	Paraoxon		Demeton-S		Malathion	
	26 ppb	*52 ppb*	*52 ppb*	*260 ppb*	*52 ppb*	*260 ppb*
Signal	15.45 ± 2.475	51.55 ± 5.445	1.1	2.7	1.9	ND
*Intensity		100%	2.1%	-	3.7%	-

*Intensity: signal intensity relative to Paraoxon. ND, not determined